English Translation
PCT REQUEST

VIII-5-1	Declaration: Non-prejudicial Disclosures or exceptions to lack of Novelty Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)): Name:	in relation to this international application Kazuo YAMAMOTO declares that the subject matter claimed in this international application was disclosed as follows:
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VIII-5-1 (iv)	Place of disclosure:	
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2001 (Heisei 13-nendo)

Summary of Master's Thesis

Department of Integrated Biosciences

February 18 to 20, 2002

Lecture Room, Frontier Life Wing at the Kashiwa Campus

Graduate School of Frontier Sciences, The University of Tokyo

14:00-14:20 Akira SATO (Laboratory of Molecular Medicine)
Establishment of glycosylation-modified cell lines overexpressing mutated intracellular lectin VIP36

14:40-15:00 Junko SHIMAUCHI (Laboratory of Molecular Medicine)
Establishment of cell line expressing various sugar chain structures by alteration of ERGIC-53

Establishment of glycosylation-modified cell line overexpressing mutated intracellular lectin VIP36

Akira Sato (06523)
Laboratory of Molecular Medicine

Among organelles in an eukaryotic cell, endopolasmic reticulum (ER) and Golgi apparatus forms the secretory pathway, where posttranslational modifications of secreted or membrane proteins are processed. Glycosylation of such proteins is one of major roles of the pathway. Recent researches revealed that lectins, a name given for the group of proteins that recognise carbohydrates, have important roles in sorting and quality control of glycoproteins in the secretory pathway. Vesicular integral-membrane protein of 36 kDa, or VIP36, is one of these lectins thought to recognise immature glycoproteins in trans-Golgi, and bring them back to the early stages of modification processes. It is, hence, expected VIP36 prevents "wrong" glycoproteins from being transported to the cell surface. Amino acid sequence of VIP36 have homology with ERGIC-53, another intracellular lectin, and BPA, a leguminous lectin. It is conceivable that introducing mutations at random into carbohydrate-recognition domain (CRD) of VIP36 might result in modified glycosylation presented on the plasma membrane. This idea may, in future, lead to engineering of novel sugar moieties of glycoproteins, which physiologically function. Here, by randomly mutating CRD, I attempted to establish cell lines that display modified oligosaccharides on the plasma membrane of MDCK cells, a well-known cells for the study of intracellular transport.

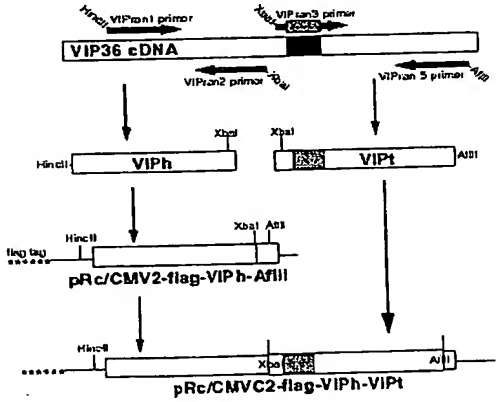

Figure. Schematic diagram of the construction of VIP36 library. Primer VIPran3 was designed with randomised oligonuculeotides (in grey rectangle on the arrrow), overlapping the carbohydrate recognition domain (CRD, in black rectangle) of VIP36. Amplified fragments will contain randomised CRD (in grey rectangle).

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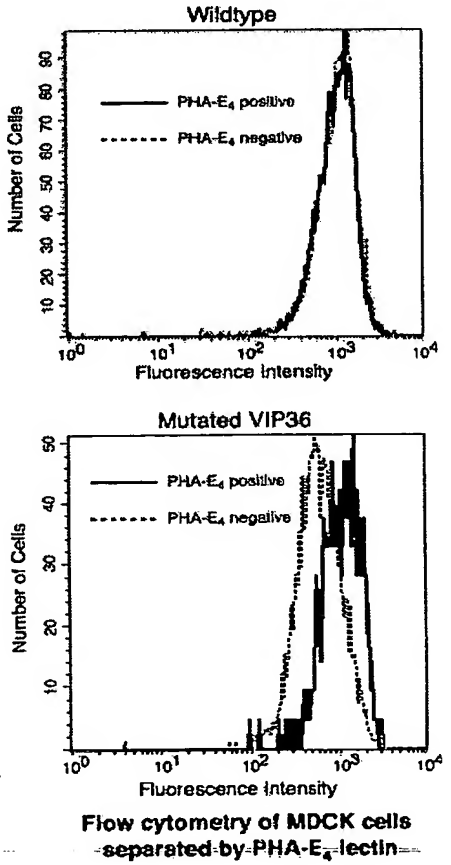
For mutated VIP36, random library of the size of 5×10^5 was constructed. In the flow cytometric analysis, most of transfectants showed no significant difference from wild type. Transfectants separated by PHA-E_4 lectin, however, were stained strongly enough for further analyses, compared with wild type cells treated in a same manner. This might indicate that the secretory pathway of the transfectants was modified by mutated VIP36, leading to a change in cell surface glycosylation.

Further studies must be required in western blot analysis and cloning of the transfectants mentioned above. Sequence analysis of mutated VIP36 within the transfectants must be carried out to identify which amino acids were responsible for the change.



Flow cytometry of MDCK cells separated by PHA-E_4 lectin

Establishment of cell line expressing various sugar chain structures by alteration of ERGIC-53

Laboratory of Molecular Medicine 06526 Junko SHIMAUCHI

Background・Purpose

When proteins synthesized in the cytoplasmic reticulum within a eukaryotic cell are subjected to oligosaccharide processing in the Golgi, the proteins are transported between cell organelles by transport vesicles. At this time, a receptor (called a cargo receptor) existing in the transport vesicle recognizes the sugar chains (carbohydrate moieties) of glycoproteins and sorts proteins to be transported outside the cell. One of such receptors, ERGIC-53, is a membrane protein existing in the transport vesicle traversing between the endoplasmic reticulum and the Golgi and having a molecular weight of 53 kDa. While most of the molecule exists inside the lumen, 12 residues on the C-terminal side exist in the cytoplasm via the transmembrane region. ERGIC-53 functions as a transporter for carrying out transport and sorting of proteins within animal cells. Since the amino acid sequence of ERGIC-53 shows high homology with those of a series of leguminous lectins, its lectin activity is attracting attention. It has also been reported that it binds to mannose in a Ca^{2+}-dependent manner. However, its detailed carbohydrate recognition specificity has not yet been analyzed.

In our laboratory, we have prepared for the first time artificial lectins having various carbohydrate specificities by modifying carbohydrate recognition domains of leguminous lectins. In a relatively small limited part of a leguminous lectin, a carbohydrate recognition domain is present with a structure such that it is sandwiched by 2 loops. It is known that one of these two loop structures greatly affects the carbohydrate recognition specificity. As described above, ERGIC-53 is a carbohydrate-recognizing

molecule having homology with leguminous lectins. Thus in this study, we have focused on the homology and constructed a mutated ERGIC-53 library by randomly mutating genes encoding loops corresponding to carbohydrate recognition domains using techniques analogous to those employed in past studies on modification of carbohydrate recognition specificity using leguminous lectin BPA. It is expected that when the library is transfected into MDCK cells and then mutated ERGIC-53 is expressed, as a result of random mutagenesis of the carbohydrate recognition domains, carbohydrate recognition specificity of mutated ERGIC-53 is modified and various cells expressing glycoproteins having various glycoforms differing from those of wild type on the cell surfaces are obtained. Our final target is to establish a cell line expressing such various glycoforms.

Method

Seven out of nine amino acids (DTFDNDGKK) corresponding to the carbohydrate recognition domain of ERGIC-53 were randomly mutated (DXXXNXXXX, where X is any amino acid) and then inserted into an expression vector, pRC-CMV2-CD8-FLAG (prepared by ligating a CD8 signal sequence and a FLAG tag (immediately following the signal sequence) to pRC/CMV2), thereby constructing a mutated ERGIC library. The carbohydrate recognition domain was randomly mutated by PCR using random primers. The mutated ERGIC library was transfected into MDCK cells. Since pRC-CMV2-CD8-FLAG has a neomycin resistance gene, selection using G418 sulfate can be carried out. Thus, after introduction of the mutated ERGIC library, MDCK cells were selected using 1.5 mg/ml G418 sulfate. For the cells obtained after selection, expression was confirmed by the Western Blotting (where an anti-FLAG antibody was used as a primary antibody and an alkaline phosphatase-labeled anti-mouse IgG

antibody was used as a secondary antibody). Furthermore, the percentage of all the MDCK cells following introduction of the mutated ERGIC library, where constant expression of the mutated ERGIC library was observed was confirmed by observation using a fluorescence microscope using the indirect fluorescent antibody method (anti-FLAG antibody and FITC-labeled anti-mouse IgG were used).

The MDCK cells following introduction of the mutated ERGIC library were sorted based on carbohydrate recognition specificity of lectins using a magnetic cell sorting and separation system (MACS). The cells were stained with 16 types of biotinylated lectins, magnetically labeled using Streptavidin microbeads, and then sorted by applying the cells to a separation column for MACS, so that flow-through fractions (hereinafter referred to as (-)) and fractions retained in the column (hereinafter referred to as (+)) were obtained. These fractions obtained following sorting were stained with various lectins, and then analyzed by a fluorescence activated cell sorter (FACS). Cell fractions separated using PNA lectins were further sorted by MACS and then analyzed by lectin staining using FACS and the Western Blotting.

Result and Discussion

We were able to construct an mutated ERGIC library with a size of 2×10^5. However, when the above random mutagenesis is carried out, an entirety of 20^7, that is, 1.28×10^9, types of amino acid sequences can be obtained. In this regard, the library size constructed herein can be said to be too small. However, we believe that in addition to aiming at establishing a cell line expressing various glycoforms, the search for a strategy for establishing such a cell line and the establishment of the cell line are also significant. Thus, we continued our study with this small-sized library.

After transfection of the mutated ERGIC library into MDCK cells and selection using G418 sulfate, expression was confirmed by the Western Blotting and then expression of the mutated ERGIC library could be confirmed. However, when the cells were observed by a fluorescence microscope using the indirect fluorescent antibody method, the percentage of the cells actually expressing randomly mutated ERGIC-53 stably was as small as 3.1% of all the cells. In this study, another purpose is to prepare a pool of cells stably expressing the mutated ERGIC library obtained by randomly mutating carbohydrate recognition domains, so as to obtain a cell line expressing glycoproteins having various carbohydrate moieties not seen in the wild type MDCK cells from the pool of the cells. Hence, there is a need to increase stable expression efficiency of a gene.

Successful sorting by MACS and obtainment of PNA(+) cells presenting on the cell surfaces carbohydrate moieties that are specifically recognized by PNA (lectin derived from peanuts) were achieved by analysis made by lectin staining using FACS (right figure). PNA is a lectin specifically bound to a oligosaccharide having a structure wherein Galβ1$\rightarrow$3GalNAc is at an unreduced end, that is, a Ser/Thr linked oligosaccharide. Accordingly, it is considered that as a result of introduction of the mutated ERGIC library, cells presenting many Ser/Thr linked oligosaccharides on cell surfaces were collected in PNA(+). It is very interesting that the wild type ERGIC-53 is a molecule recognizing mannose in a Ca^{2+}-dependent manner and its recognition specificity is greatly different from recognition specificity suggested in PNA(+).

The PNA2(+) cell fraction obtained by further sorting by MACS of PNA(+) was analyzed by lectin staining using FACS. PNA2(+) showed binding to the PNA lectin more strongly than in the case of PNA(+). In the meantime, as a result of lectin staining using the Western Blotting, almost no differences were observed between the fluorescence intensity of

PNA2(+) with the PNA lectin and that of PNA(+) with the same. This may be because intracellular glycoproteins were observed at the same time in this system. In the future, cloning of PNA2(+) and further analysis of single cell will be required.

In this study, it can be said that various problems and possibilities emerged for establishing by alteration of ERGIC-53 a cell line expressing glycoproteins having various glycoforms differing from those of wild type.

平成１３年度

修士論文要旨

先端生命科学専攻

平成１４年２月１８日－２０日
柏キャンパス新領域生命棟講義室

東京大学大学院新領域創成科学研究科

先端生命科学専攻　修士論文発表会

２月１８日（月）

---------- 休憩 ----------

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Akira Sato (06523)
Laboratory of Molecular Medicine

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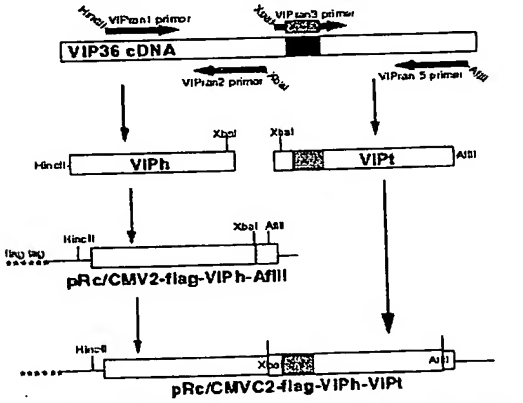

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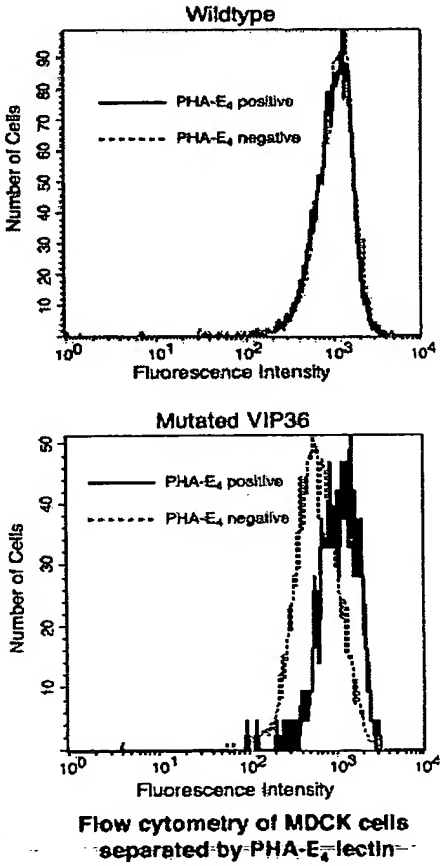


Flow cytometry of MDCK cells separated by PHA-E_4 lectin

ERGIC-53 改変による種々の糖鎖構造発現細胞株の樹立

医薬デザイン工学分野 06526 島内淳子

■背景・目的■

真核細胞内の小胞体で合成された蛋白質がゴルジ体で糖鎖のプロセシングを受ける際、輸送小胞によって細胞内小器官を行き来する。この時、輸送小胞に存在するレセプター（cargo receptor と言う）が糖蛋白質の糖鎖を認識し、細胞外に運び出される蛋白質の選別を行う。これらのレセプターのひとつである ERGIC-53 は、小胞体とゴルジ体の中間領域を行き来する輸送小胞に存在する分子量53KDa の膜蛋白質である。分子の大部分が内腔側にある一方、膜貫通領域を経て C 末端側の 12 残基が細胞質にあり、動物細胞内で蛋白質輸送選別を行うトランスポーターとして機能している。ERGIC-53 は、そのアミノ酸配列が一連の植物マメ科レクチンと高い相同性を示すことから、そのレクチン活性が注目されている。Ca^{2+}依存的にマンノースと結合するという報告もあるが、詳細な糖結合特異性の解析も未だなされていない。

当研究室において、マメ科レクチンの糖鎖認識部位を改変することにより、様々な特異性を持たせた人工レクチンの作製が初めて行われた。マメ科レクチンには比較的小さな限定された部分に、2 つのループに挟まれた構造で糖鎖認識部位が存在し、そのうちの一つのループの構造が糖結合特異性を大きく左右していることがわかっている。先に述べたとおり、ERGIC-53 はマメ科レクチンと相同性を持つ糖鎖認識分子であることから、本研究では、その相同性に着目し、すでに研究が行われたマメ科レクチン BPA を用いた糖結合特異性の改変の研究と類似の手法で、糖鎖認識部位に相当するループをコードする遺伝子をランダムに改変した ERGIC-53 改変ライブラリを作製した。これを MDCK 細胞にトランスフェクトし、改変型 ERGIC-53 を強制発現させると、糖鎖認識部位をランダマイズした影響を受け、改変型 ERGIC-53 の糖結合特異性が変化し、野生型とは異なる様々な糖鎖構造を持った糖蛋白質を細胞表面に発現している様々な細胞が得られることが期待される。このような種々の糖鎖構造発現細胞株を樹立することを最終的な目標とした。

■方法■

ERGIC-53 の糖鎖認識部位に相当する 9 アミノ酸（DTFDNDGKK）のうち、7 アミノ酸をランダマイズ（DXXXNXXXX、X は任意のアミノ酸）し、発現ベクターである pRC-CMV2-CD8-FLAG（pRC/CMV2 に、CD8 のシグナル配列、その直後に FLAG タグをつないだもの）に導入した、ERGIC 改変ライブラリを作成した。糖鎖認識部位のランダマイズはランダムプライマーを用いた PCR 法により行った。この ERGIC 改変ライブラリを MDCK 細胞にトランスフェクトした。pRC-CMV2-CD8-FLAG は、ネオマイシン耐性遺伝子を持つため、G418 sulfate による選択が行えるので、1.5mg/ml の G418 sulfate を用いて ERGIC 改変ライブラリ導入後の MDCK 細胞の選択を行った。選択後に得られた細胞に対し、Western Blotting 法による発現確認を行った（1 次抗体として anti FLAG 抗体、2 次抗体として Alkaline phosphatase 標識 Anti-mouse IgG 抗体を用いた）。また、ERGIC 改変ライブラリ導入後の MDCK 細胞のうち、全体の何%の細胞に ERGIC 改変ライブラリの恒常的な発現が見られるかを、間接蛍光抗体法を用いた蛍光顕微鏡による観察（anti FLAG 抗体、FITC 標識 anti Mouse IgG を使用）により確認した。

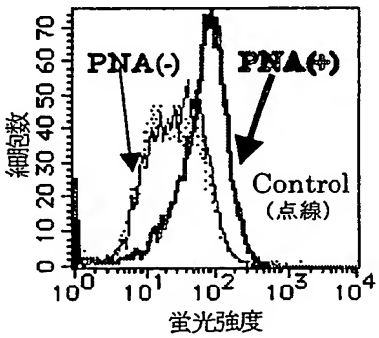
ERGIC 改変ライブラリ導入後の MDCK 細胞を、レクチンの糖結合特異性に基づき、磁気細胞分離シス

テム(MACS)を用いて選別した。16 種類のビオチン標識レクチンで細胞を染色した後、Streptavidin Microbeads を用いて磁気標識した細胞を MACS 用分離カラムにかけて選別し、素通り(以下(−)と称す)と、カラムに保持されていた画分(以下(+)と称す)を得た。これらの選別後の画分に対し、各種レクチンによる染色を行い、蛍光標示式細胞分取器 (FACS) による解析を行った。PNA レクチンで分離した細胞画分に関しては、さらに MACS による選別を行い、FACS 及び WesternBlotting 法を用いたレクチン染色による解析を行った。

■結果・考察■

ERGIC 改変ライブラリは、2×10^5 のサイズのものを作成できた。しかし、上記のランダマイズを行うと、全体で 20^7、つまり 1.28×10^9 通りのアミノ酸配列を取り得る。このことをふまえると、今回作成したライブラリのサイズは小さすぎると言える。しかし、本研究は種々の糖鎖構造発現細胞株を樹立することを目的とするのと同時に、そのような細胞株を樹立するための Strategy を模索し、確立することにも意義があると考えたので、ライブラリのサイズは小さいながらも先に進めた。

この ERGIC 改変ライブラリを MDCK 細胞にトランスフェクトし、G418 sulfate による選択の後、Western Blotting 法による発現確認を行ったところ、ERGIC 改変ライブラリの発現が確認できた。しかし、間接蛍光抗体法を用いた蛍光顕微鏡による観察を行った結果、ランダマイズした ERGIC-53 が実際に安定発現しているのは、全細胞のうち 3.1%と少なかった。本研究では、糖鎖認識部位をランダマイズした ERGIC 改変ライブラリが安定発現した細胞のプールを作成し、この中から野生型の MDCK 細胞には見られない種類の糖鎖を持った糖蛋白質が発現している細胞株を得ることを目標としている。そのためにも、遺伝子の安定発現効率を上げる必要がある。



今回 PNA (ピーナッツ由来のレクチン) が特異的に認識する糖鎖を細胞表面に提示している細胞 PNA(+)を MACS により選別・取得することが出来たことが、FACS を用いたレクチン染色による解析の結果明らかになった (右図)。PNA は Gal β 1→3GalNAc を非還元末端に有する構造の糖鎖、すなわち Ser/Thr 結合型糖鎖に対して特異的に結合するレクチンである。このことから、PNA(+)には、ERGIC 改変ライブラリを導入した結果、Ser/Thr 結合型糖鎖を細胞表面に多く提示している細胞が回収されたと考えられる。野生型の ERGIC-53 は Ca^{2+}依存的にマンノースを認識する分子であり、PNA(+)において示唆された結合特異性とは大きく異なっていることは興味深い。

PNA(+)をさらに MACS で選別することにより得られた細胞画分 PNA2(+)を、FACS を用いたレクチン染色により解析したところ、PNA(+)よりも PNA レクチンに強く結合した。一方、Western Blotting 法を用いたレクチン染色の結果、PNA2(+)と PNA(+)の PNA レクチンに対する染まり方に差はほとんど見られなかった。この系では細胞内の糖蛋白質も動じに観察したためと考えられる。今後は、PNA2(+)をクローン化し、単一細胞ごとに解析を進めることが必要であろう。

今回の研究により、ERGIC-53 の改変による、野生型とは異なる種々の糖鎖構造を持った糖蛋白質を発現している細胞株の樹立への様々な問題点と可能性が浮かび上がったと言える。

証　明　書

別添文書に記載されている論文は，平成 14 年 2 月 18 日に下記の刊行物にて発表したものであることに相違ないことを証明します。

(1)

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